(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 April 2001 (19.04.2001)

PCT

(10) International Publication Number
WO 01/27245 A2

(51) International Patent Classification[7]: C12N 5/06, 5/08, A61K 35/14, A61P 35/00

(21) International Application Number: PCT/US00/27651

(22) International Filing Date: 6 October 2000 (06.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/158,618 8 October 1999 (08.10.1999) US

(71) Applicant: DENDREON CORPORATION [US/US]; 3005 1st Avenue, Seattle, WA 98121 (US).

(72) Inventors: MONJI, Tatsue; 3545 N.E. 98th Street, Seattle, WA 98115 (US). PESHWA, Madhusudan, V.; 4034 250th Avenue S.E., Issaquah, WA 98029 (US).

(74) Agents: JUDGE, Linda, R. et al.; Iota Pi Law Group, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).

(81) Designated States *(national)*: AU, CA, JP, NZ.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/27245 A2

(54) Title: GENERATION AND CHARACTERIZATION OF A DENDRITIC CELL ISOLATED FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

(57) Abstract: A dendritic cell (DC) composition and a method of obtaining such a composition by isolating and culturing human peripheral blood mononuclear cells (PBMC) is described, together with methods of identifying and isolating DC at two different stages of maturation [the DC precursor (DCP) stage and the mature DC stage]. Also described are methods of activating T-cells employing such a DC composition.

GENERATION AND CHARACTERIZATION OF A DENDRITIC CELL ISOLATED FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Field of the Invention

The present invention relates to a dendritic cell (DC) composition, methods of isolating and culturing human peripheral blood mononuclear cells (PBMC) to obtain such a DC composition, methods of identifying and isolating DC at two different stages of maturation [the DC precursor (DCP) stage and the mature DC stage]. The invention further relates to methods of activating T-cells employing a DC composition.

Background of the Invention

Literature reports indicate that different researchers employ a diverse array of techniques for isolation and *in vitro* generation of antigen presenting cells (APC), many of which are labor intensive. There are numerous cell types that have been designated APC and protocols for isolating and activating them in *vitro* cultures. In addition, within a given APC type, there are variations with regard to isolation and activation procedures. This is particularly true with respect to DC, which function very effectively as APC.

DC have been isolated and purified using a variety of methodologies incorporating, for example, multiple-step density-gradient based isolation, monoclonal antibody panning, depletion of lineage positive cells and serum-supplemented cultures (Macatonia, S.E., *et al.*, *Immunology* 74:399-406, 1991; Markowicz, S., and Engleman, E.G., *J. Clin. Invest.* 85:955-961, 1990; Young, J.W., and Steinman, R.M., *Cell. Immunol.* 111:167-182, 1987). All the reported isolation procedures employ either sheep red blood cells and/or fetal calf serum, both of which contain potentially immunogenic foreign antigens (Ag) and/or immunogens which can interfere with the utility of the purified DC (*e.g.*, to stimulate T-cells with a selected immunogen). In summary, the prior art teaches that complex cell separation methods and cell culture in the presence of serum and cytokines are necessary for generation of mature DC.

Furthermore, there appear to be significant differences in the characteristics of the DC purified by the various prior art methods (*e.g.*, differences in cell surface marker expression). In view of these differences, it has been proposed that there exist at least three sub-types of DC (Grabbe, S., *et al.*, *Immunol. Today* 16:117-121, 1995; Thomas R., and Lipsky, P.E., *J. Immunol.* 153:4016-40128, 1994), each of which may have different properties and characteristics in terms of its Ag presenting capability.

Summary of the Invention

The invention includes, in one aspect, a method for obtaining, from a human blood sample, DC characterized by (i) a phenotype that is positive for surface Ag HLA-DR (human leukocyte Ag DR) and negative for surface antigens specific to particular cell lineages (i.e., CD3, CD14, CD16, CD19, CD20 and CD56 negative; designated herein as , "lin –"), and (ii) the ability to elicit primary and secondary immune responses when co-cultured with human lymphocytes.

According to the present invention, DCP and/or DC are obtained from peripheral blood by performing the steps of (1) standard leukapheresis; (2) buoyant density centrifugation either with a one-step or successive two-step procedure; and (3) culture of the cells *ex vivo* in serum free medium for 40 hours, in the absence of exogenously added cytokines. . DCP can be enriched prior to the 40 hour culture period by positive selection of DR+ cells using DR immunomagnetic beads or equivalent selection methods, followed by depletion of T lymphocytes, monocytes/macrophages and B lymphocytes using CD3, CD4 and CD19 immunomagnetic beads or equivalent depletion methods, respectively.

If desired, DC can also be further enriched by way of (4) post-culture enrichment by buoyant density centrifugation; and (5) depletion of monocytes/macrophages and B lymphocytes using CD14 and CD19 immunomagnetic beads with or without prior enrichment for DR+ cells. The invention includes DCP and DC obtained by the methods set forth above.

The DC of the invention is characterized functionally as capable of priming naïve helper and cytotoxic T lymphocytes and phenotypically as (1) negative for expression of lineage markers (lin -); (2) strongly positive for expression of CD 86 (CD 86++); and (3) positive for expression of HLA-DR (*i.e.*, class II MHC, DR+), designated herein as "Lin-/CD86++/DR+".

In a related aspect, the invention includes exposing DCP *in vitro* to a selected antigen during the 40 hour culture period in a manner effective to yield antigen-loaded dendritic cells.

Exemplary antigens for preparing such antigen-loaded dendritic cells include HER-2/neu and PA2024.

The invention also provides dendritic cells and/or antigen-loaded dendritic cells for use as a medicament in treating a tumor in a subject.

The invention further provides the use of a composition of dendritic cells and/or antigen-loaded dendritic cells for the manufacture of a medicament for immunizing a subject against a known tumor antigen.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 depicts the results of a mixed lymphocyte reaction (MLR), wherein various cell types within a culture of Ag-loaded mature DC were evaluated for their relative potency as stimulator cells. Cell populations analyzed include: PA undep (PA2024-loaded, undepleted, mature DC); PA-CD14 (PA2024-loaded mature DC culture from which CD14 cells were depleted); PA-CD19 (PA2024-loaded mature DC culture from which CD19 cells were depleted); and PA-CD14/CD19 (PA2024-loaded mature DC culture from which CD14 and CD19 cells were depleted). The results of ^{3}H-TdR incorporation are presented following incubation of various cell populations derived from an Ag-loaded mature DC culture (stimulators) with allogeneic T cells (responders).

Figure 2 depicts the Ag presenting capability of various populations of Ag-loaded mature DC. Cell populations analyzed are as indicated for Fig. 1, and the results of ^{3}H-TdR

incorporation are presented following incubation of each cell population (APC) with autologous T cells.

Figures 3A-D depict the results of 3-color FACS analysis of DCP at 0 hours (A, B) and 40 hours (C, D), following staining with PerCP-DR, FITC-lineage and PE-CD86 antibodies. The Lin-/DR+ cells at 0 hours shown as gated in A are weakly positive for CD86 as shown in B, while Lin-/DR+ cells at 40 hours shown as gated in C strongly express CD86 as shown in D, illustrating that DC with a Lin-/CD86++/DR+ phenotype is not present at 0 hours, but stains brightly with CD86 after 40 hours culture.

Figure 4 depicts the results of a MLR, wherein HER-2/neu-loaded mature, mock sorted DC ("BA Mock sort"), HER-2/neu-loaded APC having a traditional APC phenotype ("BA Lin+/DR+"), and HER-2/neu-loaded DC with the phenotype described herein ("BA Lin-/86++/DR+") were evaluated for their relative potency as stimulator cells. The results of ^{3}H-TdR incorporation are presented following incubation of each of the cell populations with allogeneic T cells.

Figure 5 depicts the results of a MLR, wherein: PA2024-loaded mature DC enriched by buoyant density centrifugation on BDS 56 ("PA 56I Unsorted"), PA2024-loaded APC having a traditional APC phenotype ("PA Lin+/DR+"), and PA2024 stimulated DC with the phenotype described herein. Lin-/86++/DR+ ("PA Lin-/86++/DR+") were evaluated for their relative potency as stimulator cells. The results of ^{3}H-TdR incorporation are presented following incubation of each of the cell populations with allogeneic T cells.

Figure 6 depicts the Ag presenting capability of the various cell populations described for Fig. 4, and cells cultured in the absence of Ag, with the results of ^{3}H-TdR incorporation presented following incubation of each cell population with autologous T cells.

Figure 7 depicts the Ag presenting capability of the various cell populations described for Fig. 5, and cells cultured in the absence of Ag, with the results of ^{3}H-TdR incorporation presented following incubation of each cell population with autologous T cells.

Figure 8 depicts the results of flow cytometric analysis of DCP. DCP were enriched for Lin-/DR+ cells using DR immunomagnetic beads, followed by depletion of T lymphocytes, monocytes/macrophages and B lymphocytes using CD3, CD4 and CD19 immunomagnetic beads, respectively. DCP can be characterized phenotypically as lineage negative, weakly positive for CD86 (not strongly positive) and DR positive ($Lin^{-}/86^{\pm}/DR^{+}$). The population can be enriched from approximately 0.5-1.0% (Fig. 8A, B) to approximately 50% (Fig. 8C, D), by carrying out the immunomagnetic selection and depletion steps set forth above. Under appropriate conditions, DCP differentiate into mature DC with a $Lin^{-}/CD86^{++}/DR^{+}$ phenotype (Fig. 8E, F), following co-culture for 40 hours with PBMC, which has not been subjected to the separation steps set forth above. Populations gated for Lin^{-}/DR^{+} cells in Figure 8A, C and E are shown for CD86 expression in Figure 8B, D, and F, respectively.

Figure 9 depicts the results of MLR wherein PA2024-loaded mature mock-sorted DC (PA2024 Mock-sort), PA2024-loaded FACS sorted CD54+ cells (PA2024 CD54+) and PA2024-loaded CD54- cells (PA2024 CD54-) were evaluated for their relative potency as stimulator cells. The results of ^{3}H-TdR incorporation are presented following incubation of each of the cell populations with allogeneic T cells.

Figure 10 depicts the Ag presenting capability of the various cell populations described for Figure 9 and cells cultured in the absence of Ag (w/o Ag), with the results of ^{3}H-TdR incorporation presented following incubation of each cell population with autologous T cells.

Detailed Description of the Invention

I. Definitions

Unless otherwise indicated, the terms below have the following definitions.

"Dendritic cell precursors", or "DCP", are peripheral blood cells which can mature into DC under suitable conditions. DCP typically have a non-dendritic morphology and are not competent to elicit a primary immune response as antigen presenting cells.

"Dendritic cells", or "DC" are matured DCP, which typically have a dendritic cell morphology, that is, they are large veiled cells which extend dendrites when cultured *in vitro*. When pulsed with Ag or peptide, such DC are capable of presenting Ag to naïve T cells.

"Precursor antigen presenting cells" are cells that, when exposed to an Ag or peptide, are capable of becoming APC and presenting Ag to T cells.

"Antigen presenting cells" (APC) are cells which, when exposed to an Ag or peptide, can activate $CD8^+$ cytotoxic T-lymphocytes (CTL) or $CD4^+$ helper T-lymphocytes in an immune response.

As used herein, the term "professional antigen presenting cells" includes DC, monocytes/macrophages (CD14+ cells) and B lymphocytes (CD19+ cells).

As used herein, the term "lin –" refers to a cell population that is negative for cell surface expression of the lineage markers; CD3, CD14, CD16, CD19, CD20 and CD56.

As used herein, the term "allostimulatory" means capable of stimulating allogeneic T cells due to differences in MHC molecules expressed on the cell surface.

As used herein, the terms "CD 86 bright", "CD 86++", and "strongly CD 86 positive" mean the cells are strongly immunoreactive with antibodies specific to CD 86, i.e., the results of a flow cytometry analysis of cells stained with a fluorescently labeled anti-CD86 antibody indicate a fluorescence intensity that is one log greater than that of monocyte/macrophage or B cells stained with the same anti-CD 86 antibody, using the same procedure. Such cells are said to express CD 86 at a high level on the cell surface.

As used herein, the term "Ag-loaded mature DC" refers to a PBMC-derived cell culture enriched for DCP and cultured *ex vivo* in the presence of Ag, *i.e.*, a tumor Ag. Such "Ag-loaded mature DC" include DC and various types of PBMC including professional APC such as monocytes/macrophages, which are positive for cell surface expression of CD14, and B lymphocytes, which are positive for cell surface expression of CD19.

As used herein, "immunogen" refers to a substance that is able to stimulate or induce a humoral antibody and/or cell-mediated immune response.

"Antigen" or "Ag" refers to a substance that reacts alone or in the context of MHC molecules with the products of an immune response (*e.g.*, antibodies, T-cell receptors) which have been stimulated by a specific immunogen. Antigens therefore include the specific immunogens giving rise to the response (*e.g.*, antigenic peptides, proteins or polysaccharides) as well as the entities containing or expressing the specific immunogens (*e.g.*, viruses, bacteria,

etc.).

"Tumor antigens" refer to tumor-associated Ag and tumor-specific Ag. Examples of tumor antigens include HER-2/neu, prostatic acid phosphatase (PAP) and any of a number of proteins and polysaccharides expressed by tumor cells.

As used herein, the term "mock sorted" refers to cells that have been stained with fluorescently labeled antibody and run through all the steps of a flow cytometry analysis or sorting procedure, but are not sorted.

II. Isolation And Culture Of Cells Enriched For DC

The present invention relates to the isolation, culture and characterization of DCP and DC.

The DC of the invention is not detected in freshly isolated PBMC or in fractions enriched for DCP, but appears during 40 hour *ex vivo* culture of DCP.

The DC of the invention are both allostimulatory and capable of presenting Ag such as cancer Ag to autologous T cells.

According to the methods of the present invention, such a DC population may be obtained by: (1) leukapheresis of peripheral blood; (2) buoyant density centrifugation to enrich for DCP, either with a one-step or successive two-step procedure, (using buoyant density solution BDS 77 or BDS 77 and 65, respectively; Dendreon Corp.); and (3) culture of DCP *ex vivo* in serum free medium for 40 hours, in the presence of Ag and in the absence of exogenously added cytokines.

DCP can be enriched prior to the 40 hour culture period by positive selection of DR+ cells using DR immunomagnetic beads or equivalent selection methods, followed by depletion of T lymphocytes, monocytes/macrophages and B lymphocytes using CD3, CD4 and CD19 immunomagnetic beads or equivalent depletion methods, respectively.

If desired, DC can also be further enriched by way of (4) post-culture enrichment by buoyant density centrifugation; and (5) depletion of monocytes/macrophages and B lymphocytes using CD14 and CD19 immunomagnetic beads with or without prior enrichment for DR+ cells.

It should be noted that any of a variety of gradient materials known to those of skill in the art may be used to achieve enrichment of DCP, examples of which are provided above.

Exemplary serum-free media for culturing the cell population containing DCP include Dulbecco's Modified Minimal Essential Medium (DMEM):F-12 (1:1), AIM-V, XVIVO10 XVIVO15, XVIVO20, macrophage serum-free medium, nutrient-supplemented AIM-V or Enriched Monocyte SFM (available from, *e.g.*, Gibco/BRL Life Technologies, Gaithersburg, MD). Preferably, DC are cultured in serum-free and protein-free medium.

The DCP cell population is characterized phenotypically as lineage negative, i.e., negative for expression of the cell-surface lineage markers CD3, CD14, CD16, CD19, CD20, and CD56; weakly positive for the cell surface marker CD86 (i.e., not strongly positive); and positive for cell surface expression of HLA DR (Lin^-/CD86±/DR+). Similarly, the DC population is characterized as lineage negative, strongly positive for CD86 ("CD86-bright"); and positive for cell surface expression of HLA DR (Lin-/86++/DR+). This Lin-/86++/DR+ population is not detected in freshly isolated PBMC or in cell fractions enriched for DCP.

During the 40-hour culture period DCP mature into a cell population which expresses

additional phenotypic markers known to be associated with dendritic cell function, including DC-associated costimulatory and adhesion molecules such as CD1a, CD11c, CD40, CD54, and CD80. The acquisition of the phenotype correlates with the functional maturation of DC, in that DCP become potent APC which are not only allostimulatory but also capable of presenting Ag, exemplified by prostate cancer Ag and breast cancer Ag, to autologous T cells.

FACS-sorted Lin-/86++/DR+ cells demonstrated stronger responses than cultured B cells and monocytes/macrophages in allogeneic mixed leukocyte reaction (MLR) and Ag presentation assays (Example 3). The results indicate that acquisition of the Lin-/86++/DR+ phenotype during *ex vivo* culture signals the maturation of DCP into competent DC that are capable of inducing Ag-specific immune responses.

The purity of DC in this fraction may be quantified using, for example, flow cytometry (*i.e.*, FACS) analysis, together with functional assays.

Exemplary functional assays are described below. However, it will be understood that any of a number of assay protocols routinely employed by those of skill in the art may be used to complete such analyses.

III. Characterization Of Enriched Cell Fraction

Historically DC have been characterized as negative for the cell surface markers CD3 (T-cells), CD14 (monocytes/macrophages), CD19/20 (B-cells), CD56 (NK cells) and positive for HLA class II expression. (Macatonia, *et al.*, 1991; Markowicz and Engleman, 1990; Young and Steinman, 1987).

DC are known to those of skill in the art as the most potent APC. They are the only APC capable of priming naïve helper and cytotoxic T lymphocytes. Because of this unique ability, therapeutic applications of DC have been found to be promising in the treatment of cancer patients.

DC represent a small, heterogeneous population of leukocytes found in various lymphoid and non-lymphoid organs at different differentiation/maturation stages. Identification of DC and DC lineage cells has been difficult because of their heterogeneity, scarcity in the peripheral blood and the lack of a DC-specific cell surface marker. DC have been typically identified by their characteristic dendritic morphology, potent Ag-presenting abilities, and their cell surface phenotype characterized by a panel of monoclonal antibodies (mAb) against several multi-lineage markers such as CD1a, CD11b, CD11c, CD40, CD54, CD80, CD83, CD86, CD123 and upregulated MHC molecules. (Grabbe, S., *et al.*, *Immunol. Today* 16:117-121, 1995).

Although DC are included in a population of large CD54+ cells, the major constituents of such a cell population are monocytes and macrophages. (Example 1)

As described herein, when cultured *ex vivo* in the presence of cancer Ag, DCP collected from the peripheral blood of patients differentiate into mature Ag-loaded DC, designated herein as "Lin-/86++/DR+". Such mature Ag-loaded DC may be reinfused into patients, and induce *in vivo* immune responses directed to cancer Ag.

In the methods of the present invention, DC in the Ag-loaded DC fraction typically have a dendritic morphology when cultured *in vitro*. Further, the cells are typically negative for

lineage specific cell surface markers such as CD3, CD14, CD16, CD19, CD20 and CD56; positive for MHC class II, as evidenced by HLA-DR expression; and exhibit high level expression of the cell surface marker, CD86.

CD 86, also known as "B7-2", is expressed on APC such as B cells and monocytes/macrophages. The expression of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) on APC is upregulated by the interaction between CD40 expressed on APC and CD40L expressed on T cells. CD80 and CD86 have been shown to bind to CD28 on T cells providing a costimulatory signal necessary for T cell activation. The activation of T lymphocytes in a primary response requires that APC deliver, in addition to an Ag specific signal, several costimulatory signals that work in an additive manner. Among them, costimulation via CD80/CD86-CD28 is of critical importance,

A. Biological Activity

The biological activity of various cell populations was evaluated in assays used by those of skill in the art to characterize DC. Such assays include, the mixed lymphocyte reaction or MLR, wherein irradiated stimulator cells are cultured with allogeneic T cells (responders). The cells are cultured in medium containing ^{3}H-thymidine for the final 18 h of a 6-day incubation period, with ^{3}H-thymidine incorporation measured as an indicator of responder cell proliferation. A second assay indicates the Ag presentation ability of the Ag-loaded cells based on the ability of irradiated cells to stimulate autologous T cells, measured as above for the MLR.

The results of such studies indicate that: (1) the DC population described herein as "Lin-/86++/DR+", are approximately 20-fold more stimulatory than monocytes/macrophages and B lymphocytes in an allogeneic MLR; and (2) the Lin-/86++/DR+ cells are capable of presenting tumor Ag to autologous T cells, with an Ag-presenting ability that is at least 20-fold that of monocyte /macrophages and B lymphocytes.

Together, the results indicate that the Lin-/86++/DR+ population exhibits phenotypic and functional characteristics typical of mature competent DC, suggesting that the Lin-/86++/DR+ phenotype can be used to identify mature DC.

IV. Utility

The population of cells described herein as DCP can be characterized phenotypically as Lin-/86±/DR+. The population of cells described herein as DC can be characterized phenotypically as Lin-/86++/DR+ and express all relevant molecules typically associated with DC on their cell surface. The cells are potent allostimulatory and Ag-presenting cells.

The DCP and DC phenotypes described herein find utility in a number of applications, including, but not limited to: (1) quality control of cell processing and manufacturing in *ex vivo* generation of DC from bone marrow, PBMC, or other tissues and cell lines; (2) cellular immunotherapy; (3) clinical monitoring following DCP and/or DC mobilization, DC immunotherapy and other vaccination protocols; (4) utility as a diagnostic and prognostic tool; (5) utility in identification of DC-1, DC-2, and DC-3 sub-phenotypes; (6) utility in *ex vivo* and *in vivo* generation of T cells specific to naïve or weak Ag; (7) utility in *in vitro* and *in vivo* models of disease states, e.g., rheumatoid arthritis, multiple myeloma, autoimmunity, cancer, viral,

bacterial, and fungal infections; (8) a role in generation of immune chimeras for allo- and xeno-transplantation; (9) utility as an adjunct to HSC rescue therapy for graft-versus-leukemia or suppression of graft-versus-host disease; (10) utility in gene therapy; and (11) utility in various research and development activities.

The following examples illustrate, but in no way are intended to limit the present invention.

Materials And Methods

1. Cells. PBMC were collected from patients and healthy donors by standard leukapheresis. DCP were isolated by buoyant density centrifugation either with a one-step [buoyant density solution (BDS) 77, Dendreon Corp.] or a successive two-step (BDS 77 and 65, Dendreon) procedure.

Allogeneic T cells were enriched from buffy coat preparations derived from 9 healthy donors using the two-step buoyant density centrifugation procedure followed by affinity negative selection column chromatography (R&D Systems). Autologous T cells were enriched from apheresis products using the same method.

2. Monoclonal Antibodies (mAb). The commercially available mAb used to stain DC and DCP for flow cytometry are: (1) the fluorescein (FITC) conjugated antibodies Lin 1 (BD), CD3 (BD), CD14 (BD), CD19 (BD), and IgG1 (BD); (2) the PE-conjugated antibodies CD11c (BD), CD40 (Immunotech), CD54 (BD), CD80 (Pharmingen), CD83 (Pharmingen), CD86 (Pharmingen), IgG1 (BD); and (3) the PerCP-conjugated antibodies HLA-DR (BD) and IgG2a (BD), wherein BD refers to Becton Dickinson.

3. Antigens (Ags). Two exemplary recombinant tumor Ags (Ag) evaluated herein were: (1) recombinant HER-2/neu, a fusion protein comprising a portion of the N-terminal extracellular domain and a smaller portion of the C-terminal intracellular domain of HER-2/neu and granulocyte macrophage-colony stimulating factor (GM-CSF) and (2) PA2024, a fusion protein consisting of prostatic acid phosphatase (PAP) and GM-CSF.

4. *Ex vivo* Culture Of DCP. DCP were cultured in teflon bags (American Fluoroseal) at a density of $1x10^7$/ml in AIM-V medium supplemented with 2 mM glutamine in a humidified incubator at 37°C under 5% CO_2 for 40 hours. During the culture period DCP were pulsed with Ag, recombinant HER-2/neu (20 μg/ml) or PA2024 (10 μg/ml).

5. Post-Culture Enrichment Of DC. Cells harvested after 40-hour *ex vivo* culture were further enriched for DC by buoyant density centrifugation using BDS 56 (Dendreon Corp.).

6. Flow Cytometry. Cell surface phenotype analysis was carried out using samples consisting of approximately 1-3 x 10^7 cells, which were incubated in 10% normal mouse serum in PBS for 10 min., washed in PBS and resuspended in 250-750 μl PBS. The cell suspension was then dispensed at 30 μl/well into round-bottom 96-well plates. FITC-, PE-, and PerCP-

conjugated mAb were added at 10 µl/well and cells incubated for 20 min in the dark on ice. Cells were then washed with 200 µl/well of PBS and resuspended in 400 µl sample of PBS, then analyzed by FACS Calibur (Becton Dickinson) using cells labeled with isotype-matched control Ab as a negative control.

7. Cell sorting was performed with a FACS Vantage I (Becton Dickinson) under sterile conditions. Cells labeled with FITC-Lin 1, PE-CD86, and PerCP-HLA DR were resuspended in PBS containing 2% human serum and bulk sorted at the flow rate of 5,000 - 10,000 cells/second. Sorting gates were determined using cells labeled with isotype-matched control Ab.

8. Immunomagnetic Purification. DCP and/or DC were enriched for DR positive cells (DR+) using DR immunomagnetic beads (Miltenyi). Cells were resuspenderd in PBS containing 0.5% human serum, with or without 2mM EDTA, and mixed with the bead suspension at a 4:1 ratio of cells/beads (v/v). After a 15 minute incubation at 4°C, DR+ cells are collected using column separation (Miltenyi).

9. Immunomagnetic depletion. T-lymphocytes, monocytes/macrophages and B lymphocytes were depleted from pre- and post-culture cells using CD3, CD14 and CD19 immunomagnetic beads (Dynal), respectively. Cells and beads were resuspended in PBS containing 2% human serum and mixed at a 1:5 ratio of cells:beads. After a 20 minute incubation at 4°C with gentle mixing, unadsorbed cells were collected and washed once in AIM-V.

10. MLR. Cells were irradiated at 3,000 rad and used as stimulators at 13 – $8x10^5$ cells/well in triplicate wells of round-bottom 96-well plates. As responders, $5x10^4$ allogeneic T cells were added to each well. One µCi of ^{3}H-thymidine was added to each well for the final 18 hours of a 6-day incubation period. At the end of incubation, cells were harvested and ^{3}H-thymidine incorporation measured. Wells containing either stimulators alone or responders alone served as controls.

11. Ag presentation assays. DCP were cultured *ex vivo* for 40 hours in the presence or absence of Ag. Cells were then either FACS-sorted or enriched for DC by immunomagnetic depletion of T-lymphocytes, monocytes/macrophages and B lymphocytes. Cells were then irradiated at 3,000 rad and their T cell stimulatory activity measured by incubating at 13 – $8x10^5$/well DC with $1x10^5$/well autologous T cells in triplicate wells of 96-well round-bottom palates. Proliferation of T cells was measured as above.

Example 1

Culture and characterization of Ag-loaded DC

Preparation of Ag-loaded mature DC involves the enrichment of DCP from PBMC and *ex vivo* culture of the precursors for 40 hours in serum free medium in the absence of exogenously added cytokines. The relative ability of various cell types found within a culture of

Ag-loaded mature DC to act as stimulator cells was evaluated by depleting various cell types from the culture and determining the effect of depleted and control cells in a MLR and for their ability to present Ag to autologous T cells. Monocytes/macrophages and/or B lymphocytes were depleted from the mature DC culture using CD14 and/or CD19 immunomagnetic beads (Dynal), respectively.

The allostimulatory activity of the resulting cells was then compared with that of an undepleted DC culture. The results of immunomagnetic depletion are summarized in Table I, which indicates extensive depletion of both CD14 and CD19 cells.

Table I.

Depletion	Composition of Stimulators after Depletion	
	% CD14 positive	% CD19 positive
None	7.4	7.1
CD14	0.2	4.6
CD19	9.1	0.2
CD14 and CD19	0.1	0.2

As indicated in Fig. 1, depletion of CD14 and CD19 cells moderately affected the allostimulatory activity of DC culture, indicating that the major allostimulatory activity of mature DC culture resides with the cells that are neither monocytes/macrophages nor B lymphocytes. When the ability of the various cell types derived from a culture of Ag-loaded mature DC to present Ag to autologous T cells was examined, similar results were obtained (Fig. 2).

Given that monocytes/macrophages and B cells are the only professional APC among lineage-positive cells, the results further suggest that the major allostimulatory and antigen presenting cells in the culture of Ag-loaded mature DC are lineage negative (lin -) cells.

Example 2

CD86 expression on mature DC

The pattern of CD86 expression on the surface of APC was evaluated during culture over a period of 40 hours.

DCP enriched from PBMC by buoyant density centrifugation with or without further enrichment using immunomagnetic beads, as described above, were cultured in the presence a HER-2/neu or PA2024 tumor Ag for 40 hours in serum free medium in the absence of exogenously added cytokines. During the culture period a moderate upregulation of CD86 was observed in the Lin+/DR+ cell population within the culture, the main constituents of which are monocytes/macrophages (CD14+ cells) and B cells (CD19+ cells).

The strongest expression of CD86 was observed in the Lin-/DR+ population of post-culture cells. This Lin-/CD86++/DR+ population was not detected in freshly isolated PBMC or in fractions enriched for DCP (Fig. 3A-B). These results indicate that Lin-/CD86++/DR+

population appears during the 40 hour culture period during which DC mature (Fig. 3C-D). Further flow cytometry analysis indicated that the Lin-/CD86++/DR+ population also expresses other DC-associated costimulatory and adhesion molecules such as CD1a, CD11c, CD40, CD54 and CD80. The absence of lineage markers and the coexpression of markers known to be associated with DC confirmed the Lin-/CD86++/DR+ phenotype, as a marker for mature DC.

Example 3

Biological activity of mature DC

DC are known in the art to be the most potent APC, which are allostimulatory and capable of presenting Ag to naïve T cells.

The functional capability of Lin-/CD86++/DR+ population was compared to that of Lin+/DR+ cells in the context of allogeneic stimulation and Ag presentation. The Lin+/DR+ population includes monocytes/macrophages (CD14+ cells) and B lymphocytes (CD19+ cells), two types of professional APC.

DCP were cultured in the presence of either recombinant HER-2/neu or PA2024, as described above, and the Lin-/86++/DR+ population was enriched by buoyant density centrifugation using BDS 56 (Dendreon Corp.). Cells collected from the interface were sorted by FACS, with the Lin-/86++/DR+ and the Lin+/DR+ cell populations collected.

Each FACS-sorted population was tested for allostimulatory activity in a MLR using allogeneic T cells as responders and for the ability to present tumor Ag to autologous T cells. Mock-sorted cells and cells cultured in the absence of Ag were included in the assays as control APC.

The Lin-/86++/DR+ cells, sorted after culturing with either recombinant Her-2/neu or PA2024, demonstrated allostimulatory activity that could be detected with less than 100 stimulators/well. The potency of allostimulatory ability was calculated as EC_{50}, the number of stimulators or APC required to elicit half-maximal T cell proliferation, with a lower EC_{50} indicative of a more potent APC.

Cells with a Lin-/86++/DR+ phenotype had approximately 20-fold greater allostimulatory activity than Lin+/DR+ cells, mock-sorted cells or unsorted BDS 56 interface cells (Fig. 4 and 5). Table II indicates approximate EC_{50} values for allogeneic stimulation in an MLR by cells having a Lin-/86++/DR+ or Lin+/DR+ phenotype following incubation with the BA (HER-2/neu-GM-CSF) or PA2024 (prostatic acid phosphatase-GM-CSF) fusion protein.

Table II.

Antigen	Lin-/86++/DR+	Lin+/DR+
BA Her-2/neu	$1.5X10^3$	$25X10^3$
PA2024	$1.0X10^3$	$25X10^3$

The functional capability of Lin-/CD86++/DR+ population was also compared to that of Lin+/DR+ cells in the context of autologous T cells. A FACS-sorted Lin-/86++/DR+ population

also demonstrated a potent ability to present naïve Ag. When compared for EC_{50}, Her-2/neu- and PA2024-loaded Lin-/86++/DR+ cells were at least 20-fold more stimulatory to T cells than Her-2/neu- or PA2024-loaded Lin+/DR+ cells. (Fig. 6 and 7, Table III).

Table III.

Antigen	Lin-/86++/DR+	Lin+/DR+
BA Her-2/neu	$3X10^3$	$70X10^3$
PA2024	$15X10^3$	$> 150X10^3$

Together, these results indicate that the Lin-/86++/DR+ population represent APC that are not only strongly allostimulatory but also capable of presenting naïve tumor-associated Ag.

Example 4

Enrichment of DCP and their maturation to DC

DCP were enriched from leukapheresis products by buoyant density centrifugation. Cells were further enriched for Lin-/DR+ cells using DR immunomagnetic beads for selection of DR+ cells, followed by immunomagnetic depletion of T lymphocytes, monocytes/macrophages and B cells with CD3, CD14 and CD19 beads, respectively. Figure 8 shows that pre-culture Lin-/DR+ cells are weakly positive for CD86 (not strongly positive) and the DCP population is enriched typically from about 0.5-1.0% (Fig. 8A, B) to approximately 50% (8C, D), following immunomagnetic selection/depletion. The enriched DCP mature into DC (Fig. 8E, F), after co-cultivation with bulk (unseparated) DCP for 40 hours. This population of matured DC expresses all relevant costimulatory molecules, including CD1a, CD11c, CD40, CD54 and CD80, associated with differentiated dendritic cell function.

Example 5

Biological activity of CD54+ cells

An adhesion molecule CD54 is expressed on APC including DC and plays a role in the activation of T cells by APC.

The functional capability of a CD54+ population was compared to that of a CD54- population in the context of allogeneic stimulation and Ag presentation. DCP were cultured in the presence of PA2024 at 10μg/ml (as described above), and cells were sorted by FACS with the CD54+ and CD54- populations collected. Each sorted population was tested for allostimulatory activity in MLR (Fig. 9) and for the ability to present Ag to autologous T cells (Fig. 10). Mock-sorted cells and cells cultured in the absence of Ag were included in the assays as control APC.

The PA2024-loaded CD54+ cells (PA2024 CD54+) demonstrated allostimulatory and Ag presenting activity approximately 4-fold greater than that of mock-sorted cells (PA2024 Mock-sort). The PA2024-loaded CD54- cells (PA2024 CD54-) were not active in either MLR

or Ag presenting activity. Similarly, unsorted cells cultured in the absence of Ag (w/o Ag) were not active in Ag presenting activity. These results demonstrate that the CD54+ population represents APC that are not only allostimulatory but also capable of presenting naïve tumor-associated Ag.

IT IS CLAIMED:

1. A dendritic cell (DC) composition, comprising:

peripheral blood mononuclear cells substantially pure with respect to a cell type characterized as lineage negative, CD86 strongly positive and DR positive (Lin-/CD86++/DR+).

2. A dendritic cell precursor (DCP) composition, comprising:

peripheral blood mononuclear cells substantially pure with respect to a cell type characterized as lineage negative, not strongly positive for CD86 and DR positive (Lin-/CD86±/DR+).

3. The cell composition of claim 4, where said cells are further characterized as CD1a negative, CD11c negative or positive, not strongly positive for CD40, not strongly positive for CD54 and CD80 negative.

4. A dendritic cell composition prepared by a process comprising the steps of:

(a) obtaining a blood sample from a subject;

(b) enriching said blood sample in a manner effective to obtain a population of dendritic cell precursors (DCP);

(c) culturing said DCP *ex vivo* in a manner effective to obtain a population of dendritic cells (DC), where said DC are characterized as lin-, CD 86++ and DR+ (Lin-/CD86++/DR+).

5. The cell composition of claim 4, where said enriching includes one or more of buoyant density centrifugation, immunomagnetic selection/depletion and fluoresecently activated cell sorting (FACS).

6. The cell composition of claim 4, where said culturing includes *in vitro* culture of DCP in serum free medium in the absence of exogenously added cytokines.

7. The cell composition of claim 4, where said process further comprises the steps of

(d) enriching said DC by further buoyant density centrifugation; and

(e) removing cells positive for cell surface expression of CD14 and CD19 from said DC composition; or

(f) FACS sorting for Lin-/CD86++/DR+ cells.

8. The DC composition of claim 1 or 4, where said cells are further characterized as allostimulatory and capable of antigen presentation to autologous T cells.

9. The DC composition of claim 1 or 4, where said cells are further characterized as positive for cell surface expression of CD1a, CD11c, CD40, CD54 and CD80.

10. The composition according to any one of claims 4 through 7 where said culturing

includes exposing said DCP *in vitro* to a selected antigen in a manner effective to yield antigen-loaded dendritic cells.

11. The composition according to claim 10, where said antigen is HER-2/neu.

12. The according to claim 10, where said antigen is PA2024.

13. A composition according to any one of claims 1 through 12 for use as a medicament in treating a tumor in a subject.

14. Use of a composition according to any one of claims 1 through 12 for the manufacture of a medicament for immunizing a subject against a known tumor antigen.

15. A method of obtaining a dendritic cell composition, comprising:

(a) obtaining a blood sample from a subject;

(b) enriching said blood sample in a manner effective to obtain a population of dendritic cell precursors (DCP);

(c) culturing said DCP *ex vivo* in a manner effective to obtain a population of dendritic cells (DC), where said DC are characterized as lin -, CD 86++ and DR+ (Lin-/CD86++/DR+).

16. The method of claim 15, where said enriching includes one or more of buoyant density centrifugation, immunomagnetic selection/depletion and fluorescently activated cell sorting (FACS).

17. The method of claim 15, where said culturing includes culture of DCP in serum free medium in the absence of exogenously added cytokines.

18. The method of claim 15, further comprising the steps of:

(d) enriching said DC by further buoyant density centrifugation;

(e) removing cells positive for cell surface expression of CD14 and CD19 from said DC composition; or

(f) FACS sorting for Lin-/CD86++/DR+ cells.

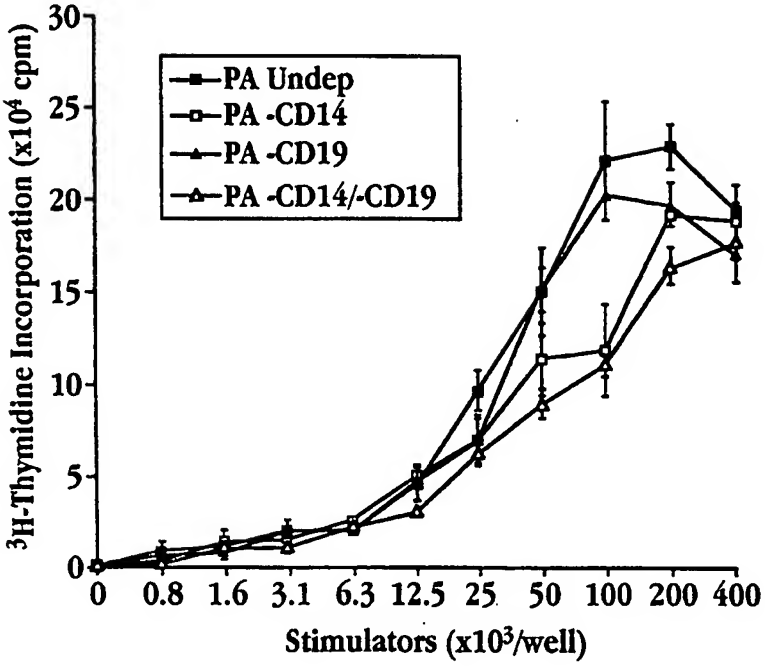
PA Undep
PA -CD14
PA -CD19
PA -CD14/-CD19
^{3}H-Thymidine Incorporation (x10^4 cpm)
0
5
10
15
20
25
30
0 0.8 1.6 3.1 6.3 12.5 25 50 100 200 400
Stimulators (x10^3/well)

Fig. 1

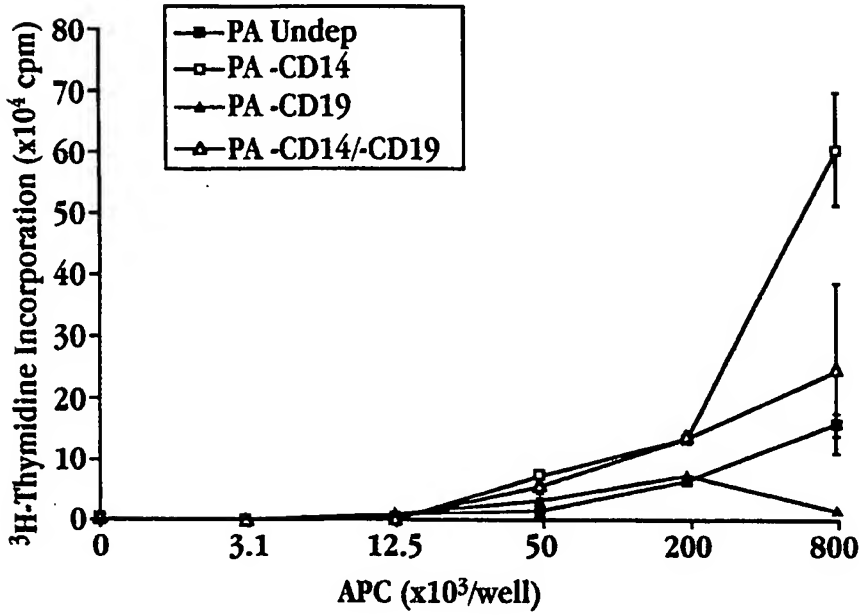
PA Undep
PA -CD14
PA -CD19
PA -CD14/-CD19
^{3}H-Thymidine Incorporation (x10^4 cpm)
0
10
20
30
40
50
60
70
80
0 3.1 12.5 50 200 800
APC (x10^3/well)

Fig. 2

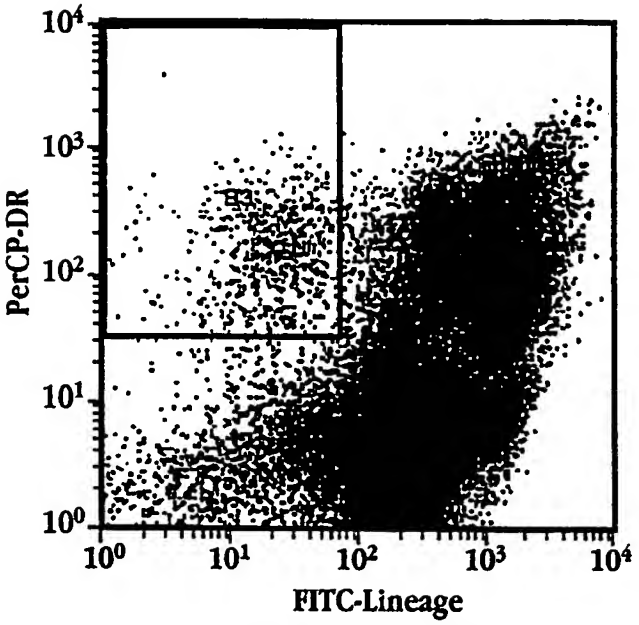
PerCP-DR
FITC-Lineage
R3
10^0
10^1
10^2
10^3
10^4

Fig. 3A

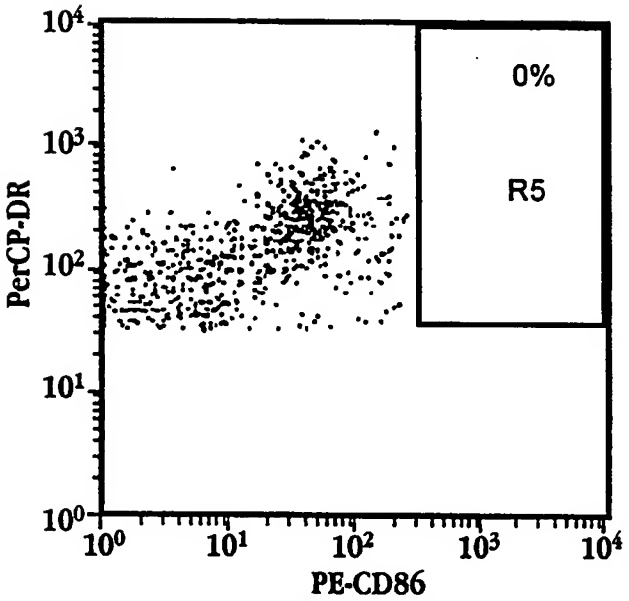
PerCP-DR
PE-CD86
0%
R5
10^0
10^1
10^2
10^3
10^4

Fig. 3B

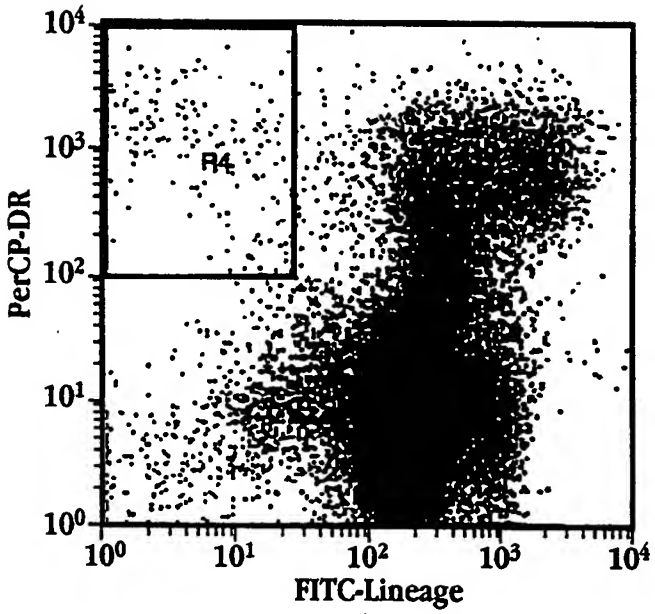
R4
PerCP-DR
FITC-Lineage
10^0
10^1
10^2
10^3
10^4

Fig. 3C

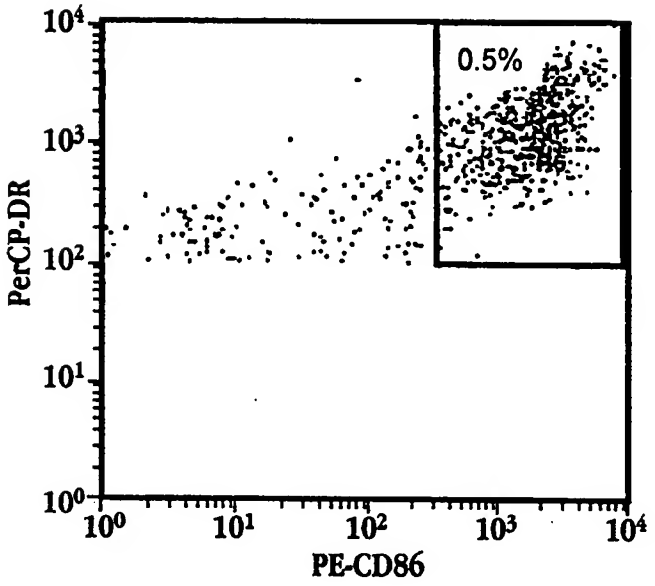
0.5%
R6
PerCP-DR
PE-CD86
10^0
10^1
10^2
10^3
10^4

Fig. 3D

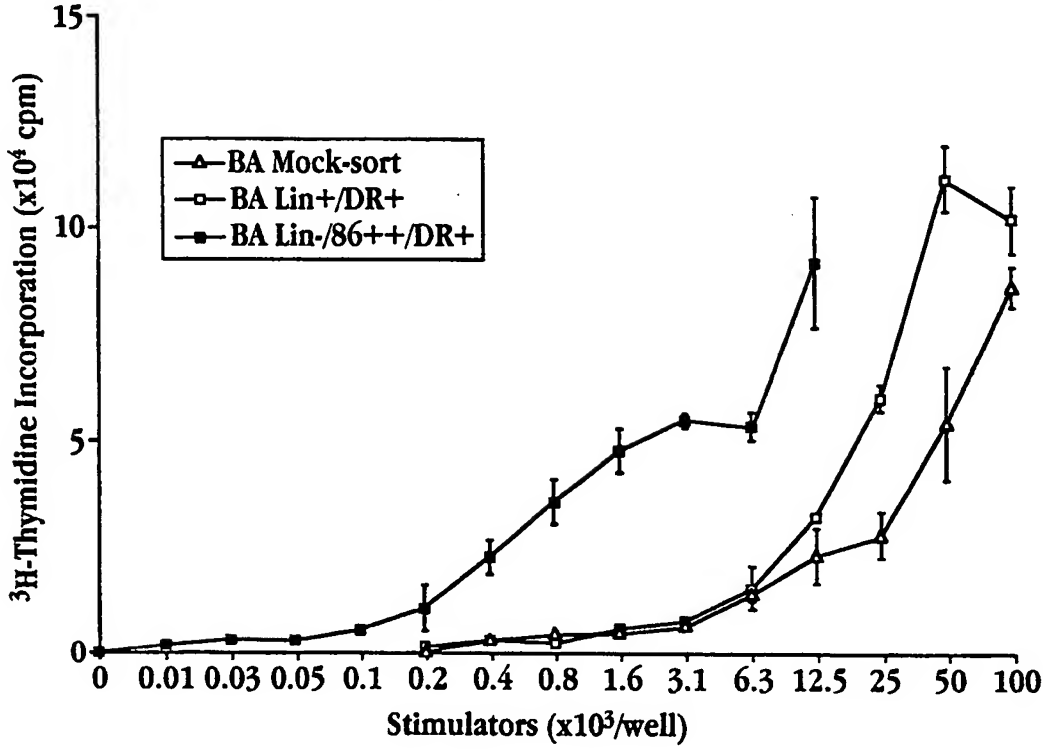
BA Mock-sort
BA Lin+/DR+
BA Lin-/86++/DR+
^{3}H-Thymidine Incorporation (x10^4 cpm)
Stimulators (x10^3/well)
0
5
10
15
0 0.01 0.03 0.05 0.1 0.2 0.4 0.8 1.6 3.1 6.3 12.5 25 50 100

Fig. 4

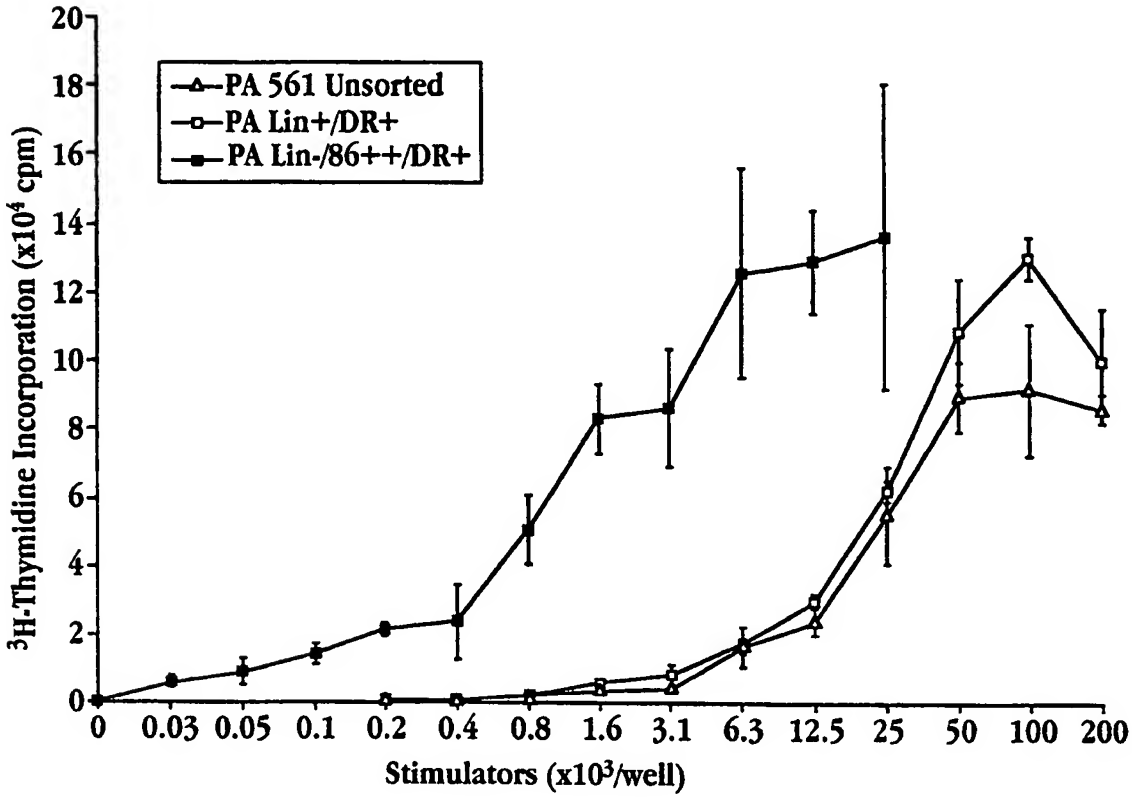
PA 561 Unsorted
PA Lin+/DR+
PA Lin-/86++/DR+
^{3}H-Thymidine Incorporation (x10^4 cpm)
Stimulators (x10^3/well)
0
2
4
6
8
10
12
14
16
18
20
0 0.03 0.05 0.1 0.2 0.4 0.8 1.6 3.1 6.3 12.5 25 50 100 200

Fig. 5

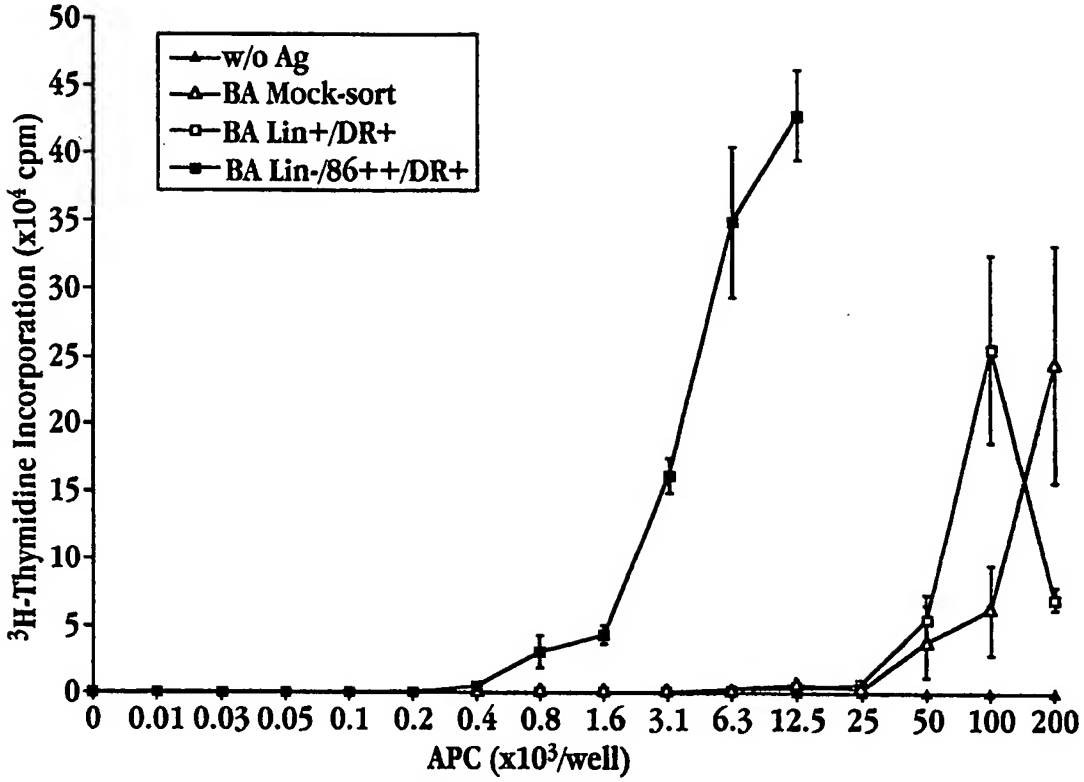
w/o Ag
BA Mock-sort
BA Lin+/DR+
BA Lin-/86++/DR+
^{3}H-Thymidine Incorporation (x10^4 cpm)
0
5
10
15
20
25
30
35
40
45
50
0 0.01 0.03 0.05 0.1 0.2 0.4 0.8 1.6 3.1 6.3 12.5 25 50 100 200
APC (x10^3/well)

Fig. 6

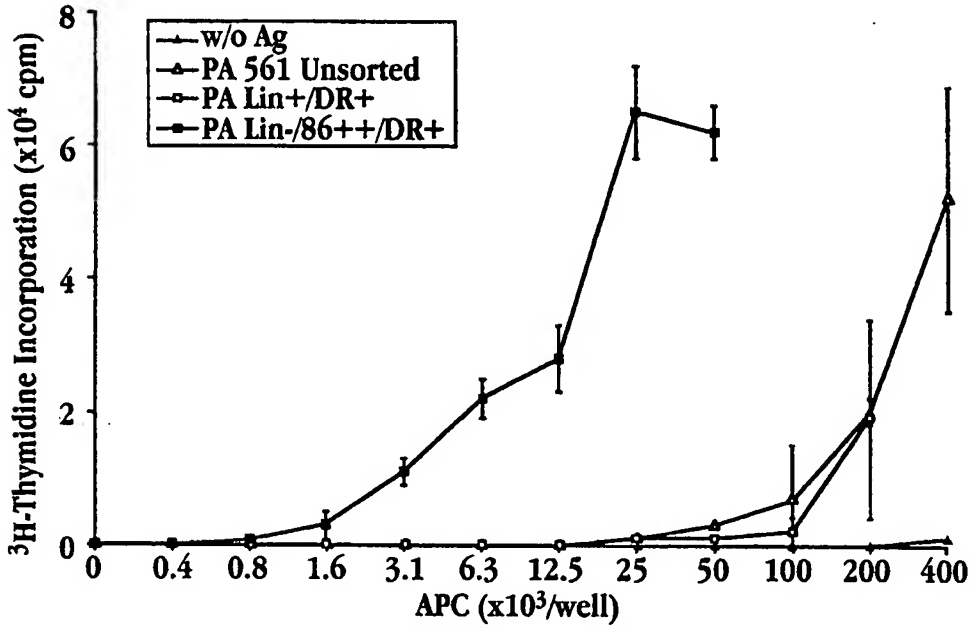
w/o Ag
PA 561 Unsorted
PA Lin+/DR+
PA Lin-/86++/DR+
^{3}H-Thymidine Incorporation (x10^4 cpm)
0
2
4
6
8
0 0.4 0.8 1.6 3.1 6.3 12.5 25 50 100 200 400
APC (x10^3/well)

Fig. 7

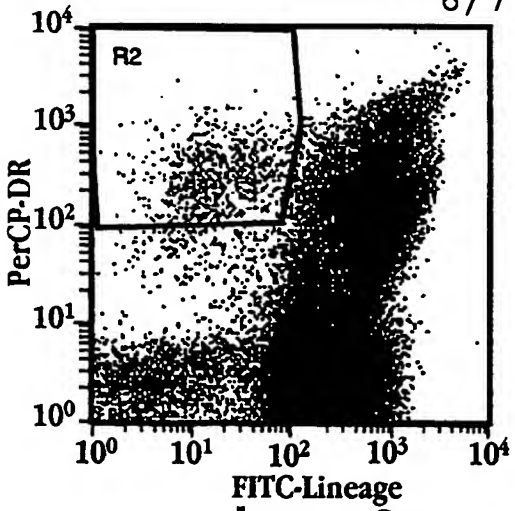

Fig. 8A

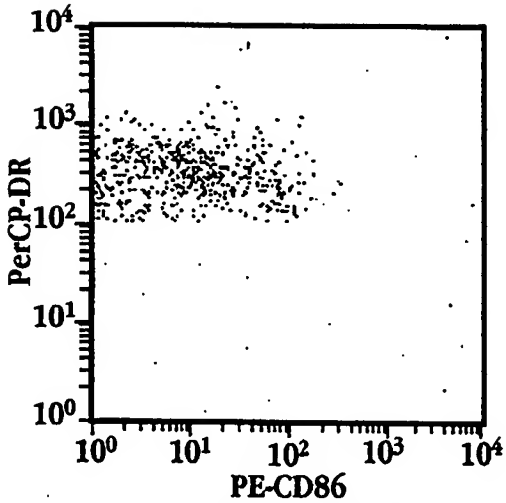

Fig. 8B

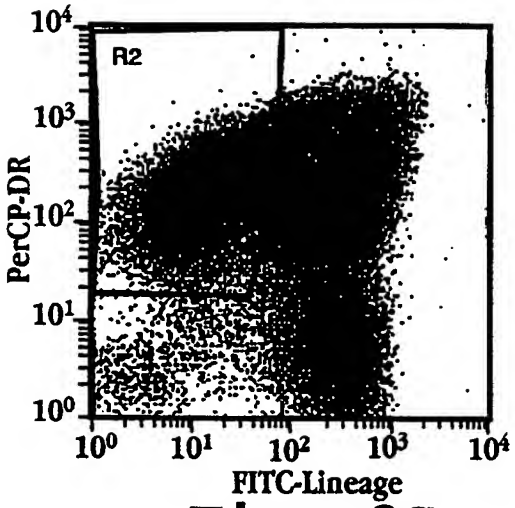

Fig. 8C

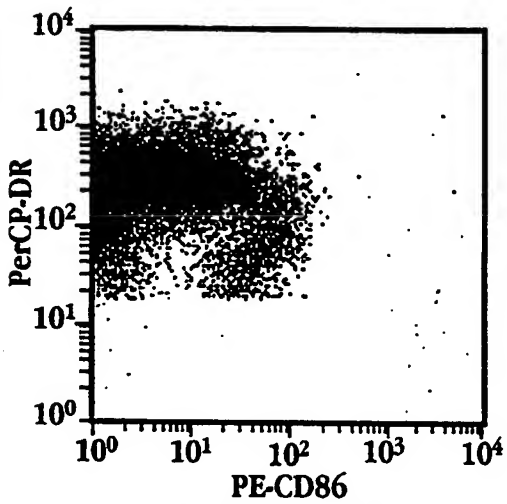

Fig. 8D

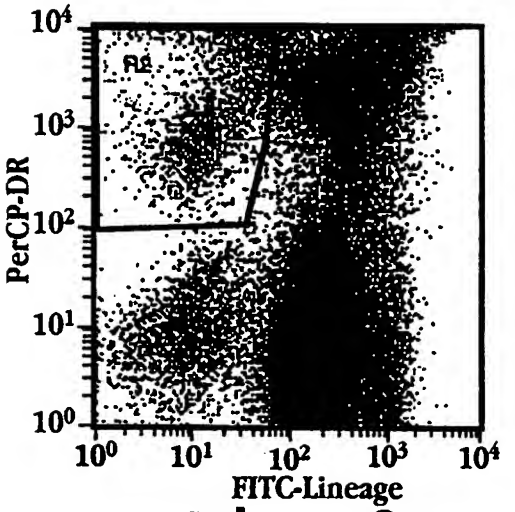

Fig. 8E

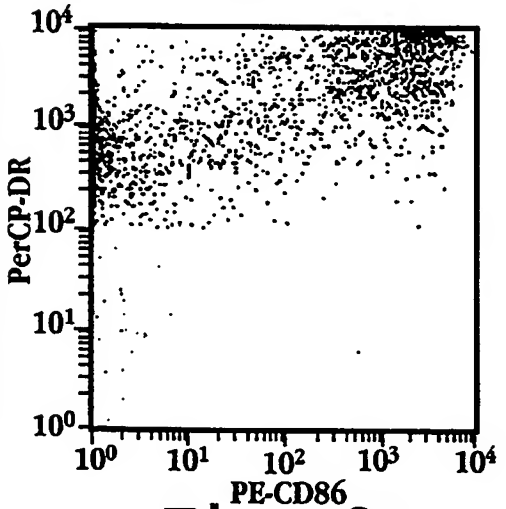

Fig. 8F

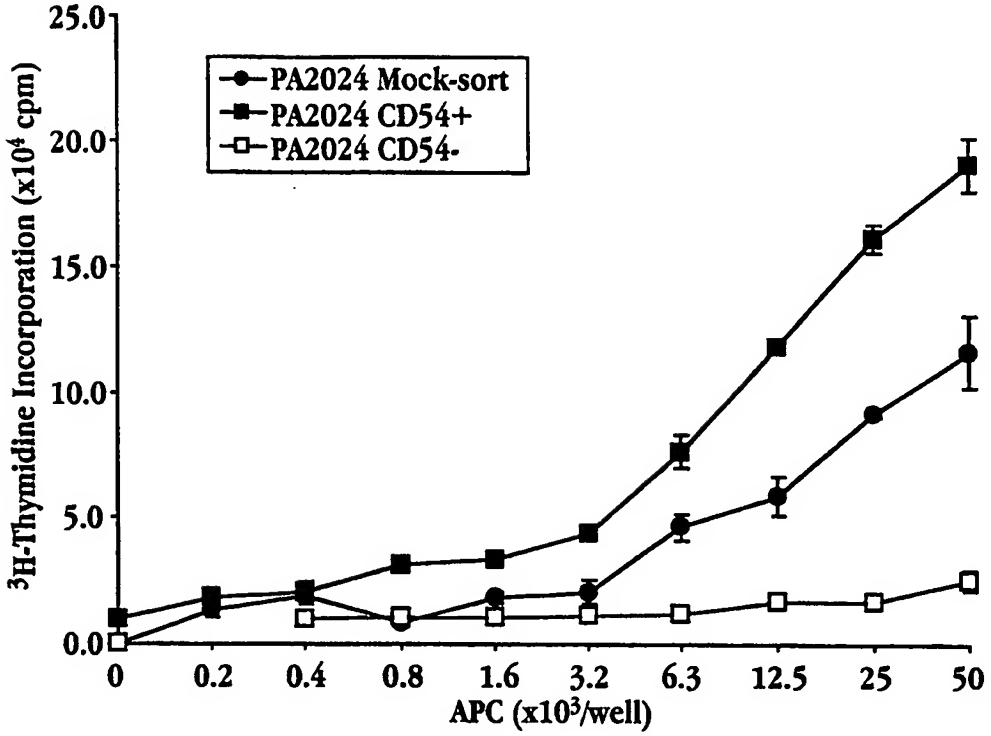
PA2024 Mock-sort
PA2024 CD54+
PA2024 CD54-
^{3}H-Thymidine Incorporation (x10^4 cpm)
25.0
20.0
15.0
10.0
5.0
0.0
0
0.2
0.4
0.8
1.6
3.2
6.3
12.5
25
50
APC (x10^3/well)

Fig. 9

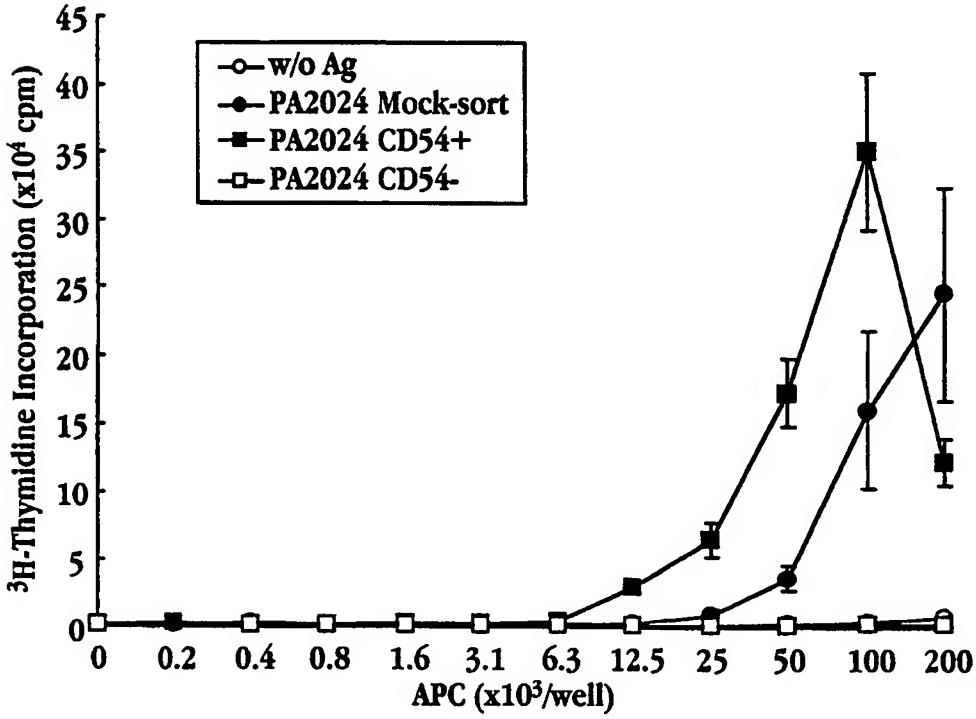
w/o Ag
PA2024 Mock-sort
PA2024 CD54+
PA2024 CD54-
^{3}H-Thymidine Incorporation (x10^4 cpm)
45
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.8
1.6
3.1
6.3
12.5
25
50
100
200
APC (x10^3/well)

Fig. 10